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Cyclooxygenase-1 Mediates the Final Stage of Morphine-Induced Delayed Cardioprotection in Concert With Cyclooxygenase-2

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OBJECTIVES We sought to investigate the time course of morphine-induced delayed cardioprotection and examine the role of cyclooxygenase (COX) in this cardioprotective effect.

BACKGROUND Cyclooxygenase-2 has been shown to be essential for the delayed cardioprotection induced by ischemic preconditioning and delta-opioid agonists.

METHODS Male mice were subjected to 45 min of coronary artery occlusion followed by 120 min of reperfusion. Expressions of COX-2 and COX-1 were assessed by Western blotting, and the myocardial prostaglandin (PG)E_2 and 6-keto-$PGF_{1\text{-alpha}}$ contents were measured using enzyme immunoassays.

RESULTS A powerful infarct-sparing effect appeared 24 and 48 h after morphine preconditioning and faded after 72 h. After 24 h, the anti-infarct effect was associated with enhanced myocardial levels of COX-2, PGE_2, and 6-keto-$PGF_{1\text{-alpha}}$, and no changes in COX-1 protein levels were found. Cardioprotection and increases in PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ were completely abolished by the COX-2–selective inhibitor NS-398 and the non-selective COX inhibitor indomethacin, whereas the COX-1–selective inhibitor SC-560 had no effect. After 48 h, up-regulation of myocardial PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ was also observed, and COX-1 expression was enhanced markedly, but only a slight increase in COX-2 expression was apparent. Cardioprotection and the increases in PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ 48 h after morphine administration were abrogated only by indomethacin, and not by SC-560 or NS-398.

CONCLUSIONS Morphine confers delayed cardioprotection via a COX-dependent pathway; COX-2 is essential for the cardioprotection observed in the initial stage (24 h), whereas, in the final stage (48 h), cardioprotection is mediated by COX-1 in concert with COX-2. (J Am Coll Cardiol 2005;45:1707–15) © 2005 by the American College of Cardiology Foundation

Ischemic preconditioning confers biphasic cardioprotection: the acute phase is limited to 1 to 3 h after a brief ischemic stimulus (1), and the delayed phase emerges 24 h later and may last up to 72 h (2,3). The biphasic cardioprotective effects of ischemic preconditioning are also mimicked by opioids (4–6), and delayed cardioprotection induced by a delta1-opioid receptor agonist has been reported to persist for up to 48 h after preconditioning (7). Morphine, the most widely used opioid, has been shown to induce the acute phase of cardioprotection in a variety of studies (4,8,9). Furthermore, our laboratory recently demonstrated that morphine can also produce delayed cardioprotection in mice 24 h after administration (10). However, the duration of morphine-induced delayed cardioprotection is still unknown.

Delayed ischemic preconditioning has been shown to be a complex process that involves a network of intricate regulatory mechanisms at the levels of cell signaling and gene expression. Convincing evidence indicates that cyclooxygenase (COX)-2 mediates the delayed cardioprotection induced by ischemic preconditioning in rabbits (3,11,12) and mice (13,14) through enhanced production of cytoprotective prostanoids such as prostaglandin (PG)E_2 and PGI_2. The COX-2 pathway is also essential for conferring the delayed cardioprotection afforded by heat stress (15) and volatile anesthetics (16), as well as by δ-opioid receptor agonists (6,17,18) in rat and rabbit models. Although divergent stimuli confer similar cardioprotective effects in different species, the molecular regulatory mechanisms involved in these effects may be different. Hence, it has been found that delayed cardioprotection induced by activation of the adenosine A1 or A3 receptors is independent of COX-2 (19). Cyclooxygenase-1, the other COX isoform, is constitutively expressed in most tissues, including heart, and has been found to be involved in gastroprotective effects induced by ischemic preconditioning (20,21). However, the role of COX-1 in cardioprotection is poorly understood. Long-term inhibition or genetic disruption of COX-1 has been reported to increase cardiac ischemia/reperfusion injury in mice (22), whereas COX-1 has been found not to mediate delayed cardioprotection induced by a delta-opioid receptor agonist in rats (6). The involvement of COX enzymes in

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Abbreviations and Acronyms

COX = cyclooxygenase
IN = indomethacin
M = morphine
NS = NS-398
PG = prostaglandin
SC = SC-560

delayed cardioprotection conferred by morphine, a non-selective opioid agonist, has not been examined in mice.

Delayed cardioprotection persists for a substantial period of time, but almost all studies of the cellular and molecular mechanisms of this event have been performed for about 24 h after the stimulus. In a recent report, it has been shown that the cardioprotection observed in the final stage (72 h) of late ischemic preconditioning is mediated by neuronal nitric oxide synthase and not by inducible nitric oxide synthase, which mediates cardioprotection in the first 24 h after ischemic preconditioning (3). These findings suggest that delayed cardioprotection is not mediated through the same mechanism throughout its duration, and that divergent mechanisms may be involved in the initial and final stages of delayed preconditioning.

In the current study, we sought to investigate the time course of morphine-induced delayed cardioprotection in a murine model of coronary artery occlusion and reperfusion and determine the potential role of COX in the initial and final stages of this cardioprotective effect.

METHODS

Animals. Male B6129PF2/J mice (body weight 25 to 35 g) purchased from Jackson Laboratory (Bar Harbor, Maine) were used in the study. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hamamatsu University School of Medicine and was in accordance with the National Institute of Health's "Guide for the Care and Use of Laboratory Animals."

Drugs and chemicals. Morphine was purchased from Shionogi Co. (Osaka, Japan). NS-398 and indomethacin were obtained from Sigma Chemical (St. Louis, Missouri), and SC-560 was purchased from Cayman Chemical (Ann Arbor, Michigan).

Surgical procedures. Myocardial ischemia was induced by coronary artery occlusion as previously described (10). The region at risk was expressed as a percentage of the weight of the left ventricle, and the infarct size was expressed as a percentage of the weight of the region at risk.

Protocol of studies for myocardial infarction. Mice were assigned to 13 groups, as shown in Figure 1. All groups were subjected to 45 min of coronary occlusion followed by 120 min of reperfusion. The first series of mice was used to define the time course of morphine-induced delayed cardioprotection (Fig. 1A). Group I (control) received saline (0.1 ml) intraperitoneally 24 h before coronary occlusion. Groups II to IV (M 24 h, M 48 h, and M 72 h) were pretreated with morphine (0.3 mg/kg) intraperitoneally 24 h, 48 h, or 72 h before induction of myocardial ischemia. The dose of morphine used in the current study has been reported to induce powerful cardioprotection in mice and rats (9,10). The second series of experiments was performed to investigate the effects of selective and non-selective COX inhibitors on the initial stage of morphine-induced delayed cardioprotection (Fig. 1B). Groups V to VII were used as drug control groups: mice received the selective COX-2 inhibitor NS-398 (5 mg/kg, group V [NS]), the selective COX-1 inhibitor SC-560 (10 mg/kg, group VI [SC]), or the non-selective COX inhibitor indomethacin (5 mg/kg, group VII [IN]) intraperitoneally 30 min before coronary occlusion. Mice in groups VIII to X were pretreated with morphine (0.3 mg/kg) intraperitoneally 24 h before coronary occlusion, and intraperitoneal injections of NS-398 (5 mg/kg), SC-560 (10 mg/kg), and IN (5 mg/kg) were administered to group VIII (M 24 h + NS), group IX (M 24 h + SC), and group X (M 24 h + IN), respectively, 30 min before coronary occlusion. A third series of experiments was performed to define the role of COX in the final stage of morphine-induced delayed cardioprotection (Fig. 1C). Mice that had been pretreated with morphine (0.3 mg/kg) 48 h earlier received NS-398 (5 mg/kg; group XI: M 48 h + NS), SC-560 (10 mg/kg; group XII: M 48 h + SC), or indomethacin (5 mg/kg; group XIII: M 48 h + IN) 30 min before coronary occlusion. NS-398, SC-560, and indomethacin were dissolved in 50% dimethyl sulfoxide (6). The dose of NS-398, SC-560, and indomethacin have previously been shown to effectively block COX-2 (6,17), COX-1 (23), and COX (22) activity, respectively.

Measurement of PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ levels. In a parallel series of experiments, hearts were collected from groups I (control), II (M 24 h), III (M 48 h), IV (M 72 h), VIII (M 24 h + NS), IX (M 24 h + SC), X (M 24 h + IN), XI (M 48 h + NS), XII (M 48 h + SC), and XIII (M 48 h + IN) (n = 4 to 5 per group) (Fig. 1). Myocardial levels of PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ (the stable metabolite of PGI_2) were determined using enzyme immunoassay kits (Cayman Chemical), as previously described (11).

Western blot analysis of myocardial COX-2 and COX-1. Additional mice from groups I (control), II (M 24 h), III (M 48 h), and IV (M 72 h) were euthanized without coronary occlusion and reperfusion (n = 5 for each group, Fig. 1). Tissue samples were homogenized in buffer A (containing 25 mmol/l Tris-HCl [pH 7.4], 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l PMSF, 1 mmol/l DTT, 25 mmol/l NaF, 1 mmol/l Na_3VO_4, and 25 μg/ml leupeptin) and centrifuged at 1,000 *g* for 10 min. The supernatant (the cytosolic fraction) was carefully removed and re-centrifuged at 16,000 *g* for 15 min to eliminate any contaminating pellet. The initial pellet was re-suspended in a lysis buffer (buffer A + 1% Triton X-100) and incubated at 4°C for 2 h. Samples were centrifuged at 16,000 *g* for 15

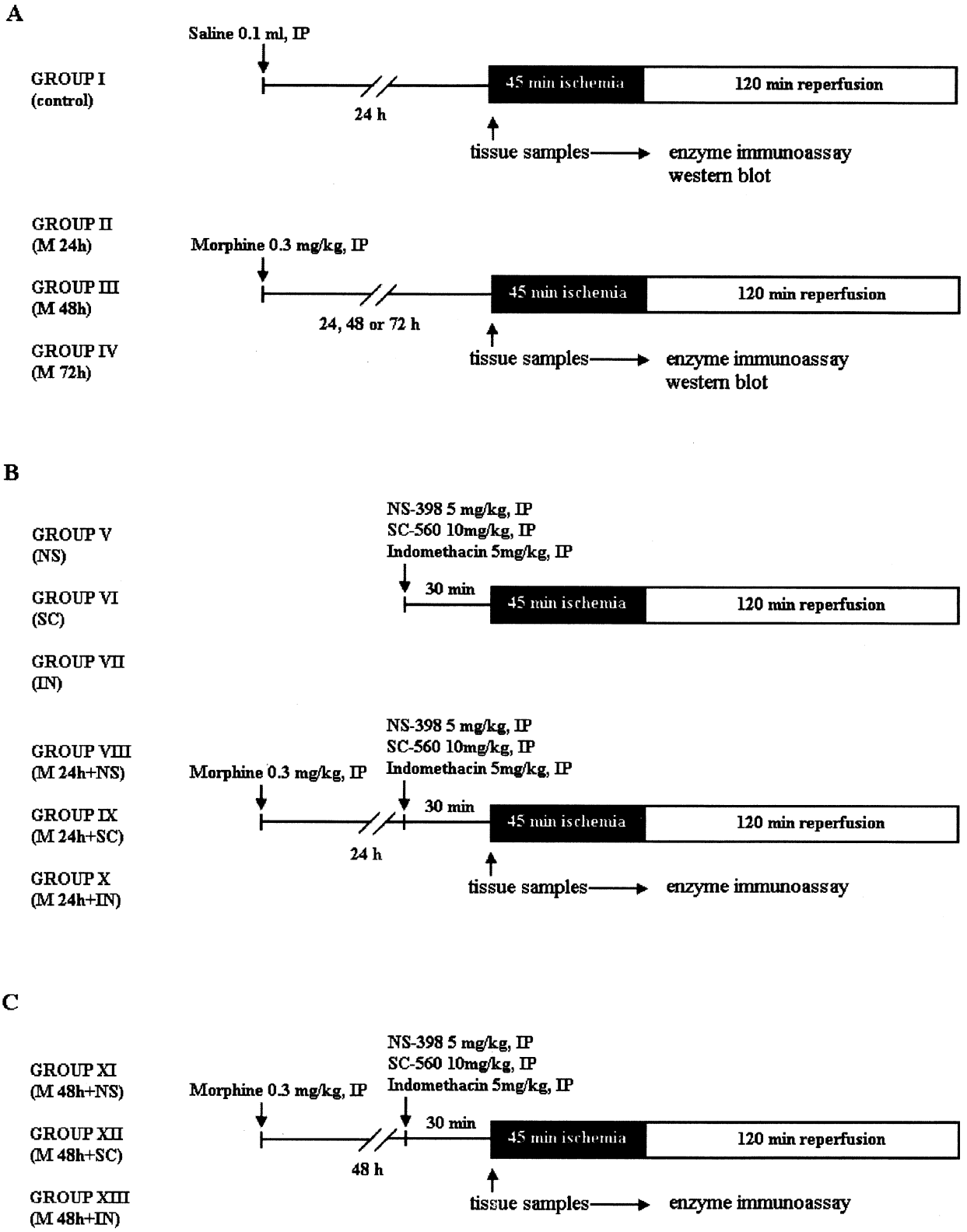

Figure 1. Experimental groups and protocol. IN = indomethacin; IP = intraperitoneal; M = morphine; NS = NS-398; SC = SC-560.

Table 1. Reasons for Excluding Mice From the Study for Infarct Size

	Group													
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	Total
Mice instrumented	10	9	10	11	9	10	9	8	9	8	11	10	9	123
Death	2	1	2	3	1	1	2		2		3	2	2	21
Technical difficulties					1	2				1		1		5
Poor staining	1				1						1			3
Final number	7	8	8	8	6	7	7	8	7	7	7	7	7	94

min. The resulting supernatants were collected as membranous fractions (6,11,24). Protein extracts were separated on 8% sodium dodecyl sulfate-polyacrylamide gels (100 μg of protein per lane) and then transferred onto a nitrocellulose membrane. Blots were blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween-20 (TBS-T) and were then washed in TBS-T. The membrane was incubated with a polyclonal rabbit antibody against mouse COX-1 or COX-2 (dilution, 1:500, Santa Cruz Biotechnology, Santa Cruz, California) at 4°C overnight. After washing with TBS-T, the membrane was incubated with an antirabbit horseradish peroxidase-linked antibody (dilution, 1:5,000, Nacalai Tesque, Kyoto, Japan) for 1 h. The membranes were developed using enhanced chemiluminescence and exposed to X-ray film for an appropriate duration. The optical density for each band on the Western blot was quantified using the National Institute of Health image program and was normalized to the corresponding Ponceau stain signal. Protein content was expressed as a percentage of the corresponding protein in controls (group I).

Statistics. All values are reported as mean ± SEM. Differences among the groups were analyzed by a one-way or a two-way repeated-measures (time and group) analysis of variance, as appropriate, followed by unpaired Student *t* tests with the Bonferroni correction. Statistical significance was defined as $p < 0.05$.

RESULTS

Mortality and exclusion. A total of 123 mice were initially included in the protocol for the infarct-size study; 29 mice were excluded for the reasons shown in Table 1.

Hemodynamics. Mean arterial blood pressure and heart rate were recorded in all groups throughout the experimental periods. The rate-pressure product (mean arterial blood pressure × heart rate/1,000) was calculated as an index of oxygen demand. Table 2 shows the hemodynamic values from all 13 groups. No significant differences in these values were observed among the groups at any time point. Coronary artery occlusion and reperfusion produced a similar increase in heart rate and similar decreases in mean arterial blood pressure and rate-pressure product in all experimental groups.

Infarct size. The myocardial region at risk, expressed as a percentage of the weight of the left ventricle, did not differ significantly among the 13 experimental groups (Fig. 2). Figure 3 summarizes the infarct size expressed as a percentage of the region at risk for each group. Infarct size was significantly smaller in mice preconditioned with morphine 24 h before induction of myocardial ischemia (group II) than in control mice (21.4 ± 2.2% vs. 43.3 ± 3.5% of the region at risk, $p < 0.001$). A similar anti-infarct effect was also observed in the mice pretreated 48 h before myocardial ischemia (group III, 24.7 ± 2.9% of the region at risk, $p = 0.003$ vs. control group). However, injection of morphine

Table 2. Hemodynamic Data

		MAP (mm Hg)			HR (beats/min)			RPP (mm Hg · min^{-1} · 1,000^{-1})		
Group	n	Baseline	Ischemia 45 min	Reperfusion 120 min	Baseline	Ischemia 45 min	Reperfusion 120 min	Baseline	Ischemia 45 min	Reperfusion 120 min
I	7	72.4 ± 3.4	62.0 ± 3.1	59.3 ± 3.8*	436 ± 13	467 ± 17	480 ± 15	31.4 ± 1.1	28.9 ± 1.8	28.6 ± 2.3
II	8	69.9 ± 3.9	63.0 ± 2.9	60.3 ± 3.5	446 ± 13	455 ± 16	476 ± 14	30.9 ± 1.3	28.5 ± 1.2	28.8 ± 2.1
III	8	76.9 ± 4.5	66.4 ± 4.4	62.4 ± 4.3	418 ± 12	440 ± 18	464 ± 16	31.9 ± 1.4	29.5 ± 2.7	28.9 ± 2.0
IV	8	66.3 ± 4.7	64.4 ± 3.9	58.8 ± 4.2	444 ± 16	452 ± 21	481 ± 14	29.2 ± 1.9	29.4 ± 2.7	28.1 ± 1.9
V	6	68.2 ± 6.0	65.0 ± 4.9	56.7 ± 4.8	463 ± 22	468 ± 25	495 ± 15	31.1 ± 1.9	30.7 ± 3.3	28.0 ± 2.4
VI	7	69.8 ± 5.6	63.7 ± 3.6	56.0 ± 3.8	437 ± 23	456 ± 24	487 ± 15	30.0 ± 1.5	29.3 ± 2.7	27.3 ± 2.0
VII	7	74.3 ± 4.4	68.6 ± 2.2	60.9 ± 5.1	428 ± 22	449 ± 24	481 ± 21	31.4 ± 1.3	30.9 ± 2.2	29.2 ± 2.5
VIII	8	70.5 ± 3.6	64.1 ± 3.0	53.9 ± 4.0*	458 ± 21	465 ± 18	497 ± 16	32.1 ± 1.7	29.9 ± 2.1	26.8 ± 2.1
IX	7	72.3 ± 3.9	65.0 ± 3.4	58.6 ± 4.6	448 ± 19	472 ± 18	501 ± 19	32.3 ± 1.9	30.7 ± 2.1	29.5 ± 2.6
X	7	70.1 ± 3.6	66.7 ± 3.5	58.6 ± 4.6	442 ± 21	468 ± 16	487 ± 14	31.0 ± 2.1	31.2 ± 1.8	28.7 ± 2.6
XI	7	73.0 ± 3.4	64.9 ± 2.8	61.0 ± 4.7	434 ± 18	484 ± 19	495 ± 12	31.6 ± 1.9	31.5 ± 1.8	30.4 ± 2.7
XII	7	68.1 ± 2.7	62.1 ± 2.5	57.7 ± 4.4	431 ± 17	463 ± 18	469 ± 16	29.2 ± 0.7	28.6 ± 0.9	27.1 ± 2.3
XIII	7	73.4 ± 4.1	66.1 ± 4.0	59.4 ± 4.2	430 ± 20	453 ± 19	500 ± 18	31.1 ± 1.1	29.6 ± 1.2	29.7 ± 2.2

Values given as mean ± SEM. *$p < 0.05$ vs. baseline.
HR = heart rate; MAP = mean arterial blood pressure; n = number of animals; RPP = rate-pressure product (MAP × HR/1,000).

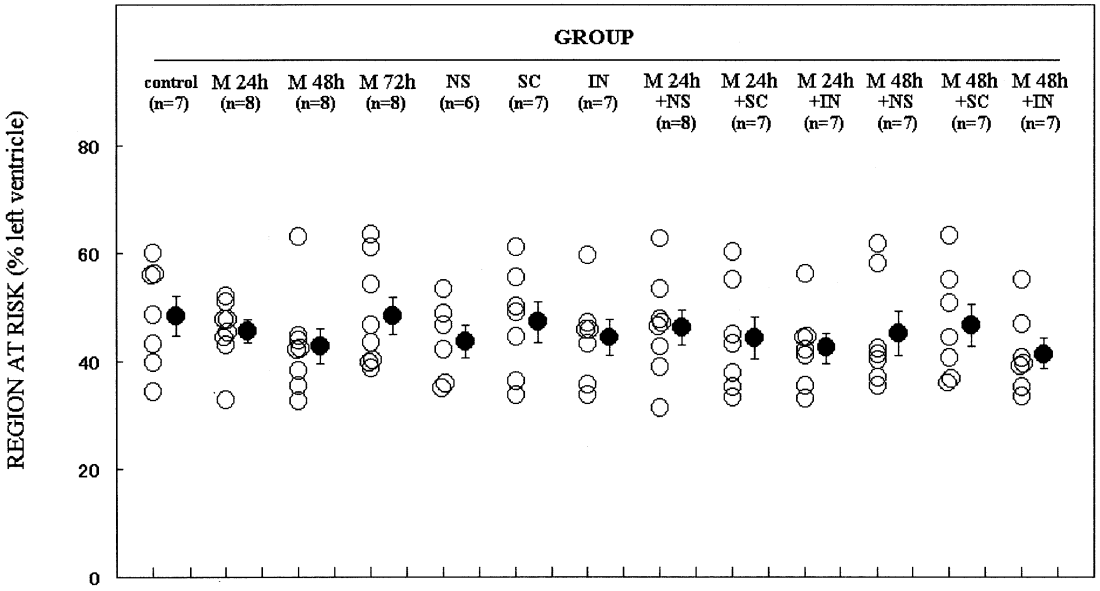

Figure 2. Measurement of myocardial region at risk in all experimental groups. The region at risk is expressed as a percentage of the weight of left ventricle. **Open circles** represent individual mice, whereas **solid circles** represent mean ± SEM. There was no significant difference among the 13 groups. IN = indomethacin; M = morphine; NS = NS-398; SC = SC-560.

72 h before coronary occlusion did not reduce the infarct size (group IV, 45.9 ± 4.3%, $p > 0.05$ vs. control group). The infarct-sparing effect 24 h after morphine pretreatment was completely abolished by the COX-2 selective inhibitor NS-398 (group VIII, 47.0 ± 2.0% of the region at risk, $p < 0.001$ vs. group II) and by the non-selective COX inhibitor indomethacin (group X, 47.1 ± 2.7% of the region at risk, $p < 0.001$ vs. group II) when the drugs were injected 30 min before coronary occlusion. However, administration of the COX-1 selective inhibitor SC-560 did not block the cardioprotection of this time point (group IX, 22.0 ± 3.8% of the region at risk, $p > 0.05$ vs. group II). Unexpectedly, both NS-398 and SC-560 failed to block the cardioprotection observed 48 h after morphine pretreatment (group XI, 26.1 ± 3.4% of the region at risk and group XII, 27.2 ± 2.4% of the region at risk), whereas indomethacin abolished such infarct-sparing effect completely (group XIII, 47.5 ± 3.7% of the region at risk, $p < 0.001$ vs. group III). Administration of NS-398, SC-560, or indomethacin did not in itself affect the infarct size in the current model.

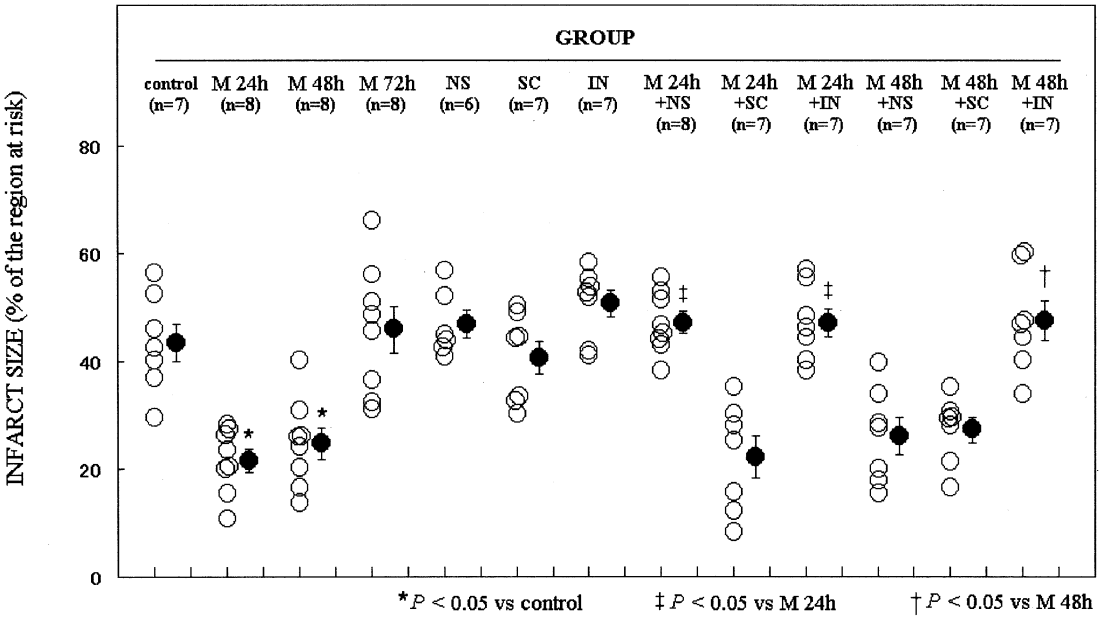

Figure 3. Measurement of myocardial infarct size in all experimental groups. The infarct size is expressed as a percentage of the region at risk. **Open circles** represent individual mice, whereas **solid circles** represent mean ± SEM. IN = indomethacin; IP = intraperitoneal; M = morphine; NS = NS-398; SC = SC-560.

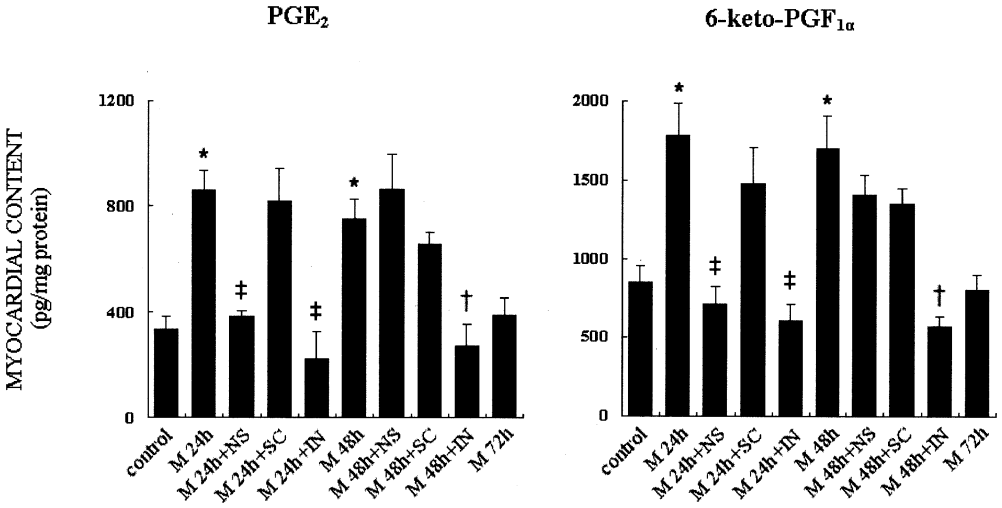

Figure 4. Myocardial content of prostaglandin (PG)E_2 and 6-keto-$PGF_{1\text{-alpha}}$ after morphine (M) pretreatment. Data are mean ± SEM. IN = indomethacin; NS = NS-398; SC = SC-560. *p < 0.05 vs. control; †p < 0.05 vs. M 48 h; ‡p < 0.05 vs. M 24 h.

Myocardial content of prostanoids. Morphine preconditioning resulted in robust increases in myocardial PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ levels 24 h (259 ± 23% and 210 ± 25% of control, respectively, p = 0.003 and p = 0.008) and 48 h later (227 ± 22% and 200 ± 26% of control, respectively, p = 0.039 and p = 0.023), compared with control mice (Fig. 4). In contrast, neither the PGE_2 nor the 6-keto-$PGF_{1\text{-alpha}}$ level was elevated 72 h after morphine administration; 24 h after morphine pretreatment, the increases in PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ were completely abrogated by administration of NS-398 or indomethacin 30 min before euthanasia, whereas SC-560 failed to block the increases of PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ at this stage. Importantly, only administration of indomethacin, but not administration of NS-398 and SC-560, completely abolished the increases in myocardial PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ levels 48 h after morphine preconditioning.

Expression of COX-2 protein and COX-1 protein. Most of the COX proteins were expressed in the membranous fraction, as reported previously (11,25,26). As illustrated in Figure 5A, a weak COX-2 signal was detected in control hearts. The expression of COX-2 protein increased markedly 24 h after morphine administration (295 ± 39% of control, p = 0.001) (Fig. 5B), and COX-2 expression was still higher than in control mice 48 h after morphine administration, although the difference was not statistically significant (161 ± 32% of control, p = 0.865). No marked change in COX-2 expression was observed 72 h after morphine preconditioning, compared with control mice.

Myocardial expression of COX-1 protein was present in control mice at a low level and did not change significantly in morphine-preconditioned mice after 24 and 72 h. Surprisingly, significant up-regulation of COX-1 protein was evident 48 h after morphine preconditioning (167 ± 26% of control, p = 0.023). Representative Western blots and the densitometric quantification of COX-1 expression are shown in Figure 6.

DISCUSSION

The salient findings of the current study can be summarized as follows: 1) morphine induces powerful cardioprotection against myocardial infarction 48 h later in mice, similarly to that observed 24 h after morphine administration, but such cardioprotection disappears 72 h after morphine pretreatment; 2) COX-2 plays an obligatory role in mediating the initial stage (24 h after morphine administration) of morphine-induced delayed cardioprotection, and the cardioprotective effect of this stage is independent of COX-1; and 3) the final stage (48 h after morphine administration) of morphine-induced delayed cardioprotection is mediated by COX-1 in concert with COX-2.

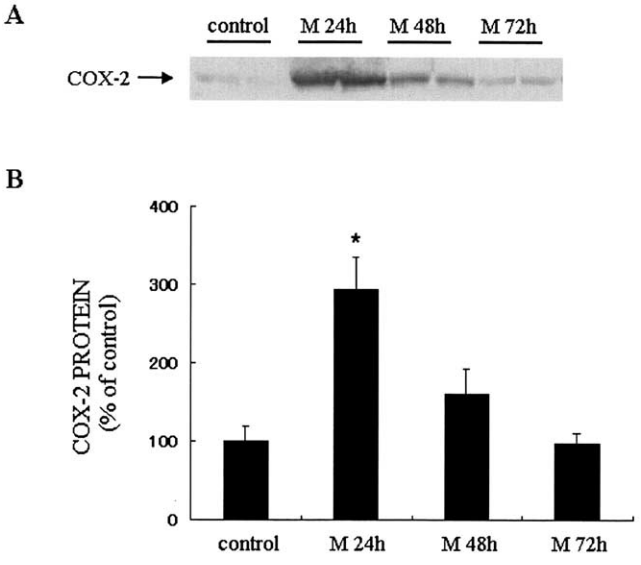

Figure 5. Expression of cyclooxygenase (COX)-2 in the membranous fraction of myocardium. **(A)** Representative Western blots illustrating COX-2 expression. **(B)** Densitometric quantification of COX-2 protein expressed as a percentage of the average value of control mice. Data are mean ± SEM. M = morphine. *p < 0.05 vs. control.

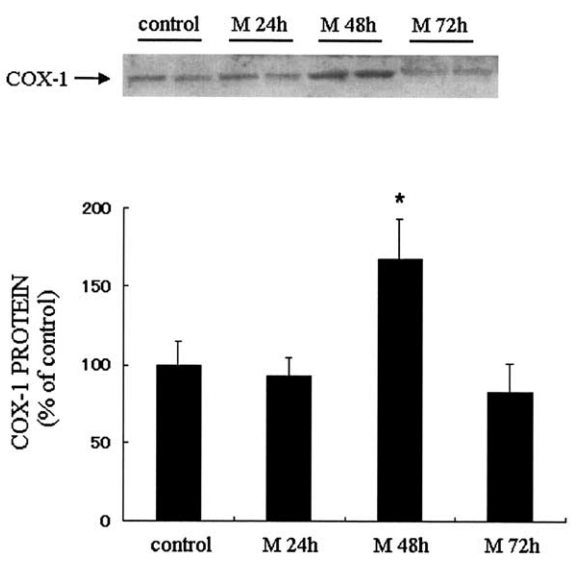

Figure 6. Expression of cyclooxygenase (COX)-1 in the membranous fraction of myocardium. **(A)** Representative Western blots showing COX-1 expression. **(B)** Densitometric quantification of COX-1 protein expressed as a percentage of the average value of control mice. Data are mean ± SEM. M = morphine. *$p < 0.05$ vs. control.

Time course of morphine-induced delayed cardioprotection. Delayed cardioprotection produced by delta-opioid agonists has been described clearly in several rat models (5–7,17,18). Our laboratory has previously demonstrated that morphine, a μ-opioid receptor agonist with δ-opioid receptor properties (27), produces delayed cardioprotection in mice (10). In the current study, myocardial infarct size as a percentage of the region at risk was significantly decreased 48 h after morphine preconditioning, as observed for a delay of 24 h after morphine administration. The anti-infarct effect was lost with a 72-h delay. These data are consistent with the observations of Fryer et al. (7), who showed in a rat model that a δ-opioid agonist can induce delayed cardioprotection beginning 24 h after a single pretreatment and fading after 72 h. However, it has also been suggested that delayed ischemia preconditioning may last for at least 72 h (2,3,28).

Role of COX in morphine-induced delayed cardioprotection. Cyclooxygenase is the rate-limiting prostanoid synthase, and it regulates the synthesis of prostanoids by catalyzing the conversion of arachidonic acid to PGH_2, the common precursor of bioactive prostanoids (29,30). Two distinct COX isoforms have been characterized: COX-1 is responsible for constitutive prostanoid formation, whereas COX-2 is induced in response to stress, but is also constitutively expressed in some tissues (29,30). Accumulating evidence has shown that COX-2 plays an essential role in conferring delayed cardioprotection induced by divergent pathophysiological stimuli or pharmacological agents. Up-regulation of COX-2 appears to be necessary for the development of delayed cardioprotection 24 h after an ischemic preconditioning stimulus (3,11,12,14), heat stress (15), and δ-opioid agonist administration (17). Although other similar studies have reported no changes in the level of COX-2 protein, these studies have shown that the late phase of cardioprotection induced by a δ-opioid agonist (6) or volatile anesthetics (16) is completely abolished by selective inhibition of COX-2. In contrast, COX-2 does not contribute to the delayed cardioprotection induced by adenosine A1 and A3 receptor agonists (19) and by acute systemic hypoxia (31). In the present study, the infarct-sparing effect and the increases in myocardial PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ contents 24 h after morphine administration were completely abolished by the COX-2 selective inhibitor NS-398 and the nonselective COX inhibitor indomethacin, whereas the COX-1 selective inhibitor SC-560 did not affect the cardioprotective effect and the increases in prostanoids. Corroborating this finding, only the expression of COX-2 protein, but not COX-1, was found to be up-regulated 24 h after morphine administration. These data suggest that COX-2 plays a key role in cardioprotection 24 h after morphine preconditioning, while COX-1 is not involved. In line with this result, it has been reported that COX-1 does not mediate delayed cardioprotection 24 h after δ-opioid agonist preconditioning, whereas COX-2 was found to do so (6).

Because morphine-conferred delayed cardioprotection lasted for 48 h in our study, we also investigated the role of COX-2 at this stage. Surprisingly, pharmacological inhibition of COX-2 or COX-1 alone failed to block the anti-infarct effect in the final stage (48 h) after morphine preconditioning. In contrast, cardioprotection was completely abolished by the nonselective COX inhibitor indomethacin. Only a slight up-regulation of COX-2 expression was observed at this stage, but COX-1 expression was enhanced markedly, and there were also marked increases of PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ in the myocardium. Furthermore, the changes of myocardial prostanoids were completely blocked by administration of indomethacin, but not by NS-398 or SC-560. These findings suggest that the signaling pathway responsible for morphine-induced delayed cardioprotection differs in the initial (up to 24 h) and final stages. Hence, both COX-1 and COX-2 are involved in the final stage of morphine-induced delayed cardioprotection in mice. When COX-2 is inhibited pharmacologically, cardioprotection and the increases in PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ levels observed 48 h after morphine administration may be generated primarily via enhancement of COX-1. We note that COX-1 has been shown to be cardioprotective and to generate PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ in COX-2 gene knockout mice (22). Although expression of COX-2 was not enhanced significantly 48 h after morphine administration, it may still be the main contributor to the infarct-sparing effect and the increases in PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ when COX-1 is blocked, particularly because COX-2 function can be enhanced without an increase in expression level (6,16). In contrast to our results, Wang et al. (3) have shown that COX-2 is essential over the whole period of delayed ischemic precon-

ditioning, although the initial and final stages of this process are mediated by different mechanisms, a discrepancy that may be due to the different stimuli and spices used in the two studies.

To assess COX enzymatic activity, the myocardial levels of PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ (the stable metabolite of PGI_2) were measured. Both PGI_2 and PGE_2 have been reported to have cardioprotective properties against ischemia and reperfusion injury (32–35). In the current study, myocardium PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ levels were enhanced significantly 24 and 48 h after morphine preconditioning, and faded after 72 h, consistent with the time course of morphine-induced delayed cardioprotection. Furthermore, the increase of PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ could be blocked by pharmacological inhibition of COX. In agreement with these findings, enhanced levels of myocardial PGE_2 and 6-keto-$PGF_{1\text{-alpha}}$ have been found to accompany delayed cardioprotection induced by ischemic preconditioning (3,11,12,14) and δ-opioid receptor agonists (6,17). Prostaglandins are also involved in acute cardioprotection conducted by ischemic preconditioning (36) and angiotensin II type 1-receptor antagonist (37); PGE_2 and PGI_2 may be mediators that activate ATP-sensitive potassium (K_{ATP}) channels (38,39), which are essential for mediation of opioid-induced cardioprotection (4,7,9,40,41). Therefore, it is plausible that morphine confers delayed cardioprotection by enhancing the synthesis of COX-dependent cardioprotective prostanoids, although it also remains possible that morphine induces cardioprotective effects via altered expression of certain proteins that are modulated by COX products.

Conclusions. In conclusion, the present study provides novel insights into morphine-induced delayed cardioprotection and the underlying molecular mechanisms. Our findings demonstrate that the anti-infarct effect induced by morphine persists 48 h after preconditioning. Cardioprotection observed 24 h after morphine administration is associated with and mediated by up-regulation of COX-2. Expression of COX-1 is enhanced 48 h after morphine preconditioning and COX-1, in concert with COX-2, mediates cardioprotection at this stage. To our knowledge, this is the first report in which COX-1 has been shown to be up-regulated and involved in delayed cardioprotection. These findings suggest that morphine-induced delayed cardioprotection is COX-dependent and that the molecular mechanism of cardioprotection differs 24 h and 48 h after morphine preconditioning. Our observations also expand the understanding of the cardioprotective properties of COX-1.

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